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(54) Title: PROTEINASE INHIBITORY AGENTS AND METHODS FOR THEIR USE

(57) Abstract

A plant-specific phytochemical isolated from cabbage and having a specific inhibitory response directed to proteinases found in *Trichoplusia ni* and *Pieris rapae* larvae including the use of the phytochemical as a means to protect the plant from these larvae is described.

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PROTEINASE INHIBITORY AGENTS AND METHODS FOR THEIR USE

The herbivorous insects which are major pests for New York State cabbage growers are the flea beetles (*Phyllotreta cruciferae*), diamondback moth (*Plutella xylostella*), imported cabbage worm (*Pieris rapae*), and the cabbage looper (*Trichoplusia ni*). Of these pests, the imported cabbage worm and cabbage looper attack the plant during middle maturation, that is when the plants reach the 6 to 8 leaf stage of growth, at a time when the grower is most susceptible to a total loss of the cabbage crop. If the plants are destroyed at this stage of maturation, there is little chance of a second replanting during the growing season. It is important, therefore, that means be found to combat these pests which are agriculturally and environmentally acceptable.

Current literature supports the hypothesis that plant proteinase inhibitors - protein or polypeptides which occur in a wide variety of plants - provide the plant with significant protection against herbivorous insects. The proteinase inhibitors which have been most extensively studied are those that inhibit serine proteinases (e.g. trypsin, chymotrypsin) that are common digestive enzymes in animals but are not present in plants. Since plants do not contain serine proteinases, the presence of serine proteinase

inhibitors in plants suggests that they are acting as a defense against herbivorous insects.

The studies suggesting the protective nature of serine proteinase inhibitors in plants originate from experiments based upon (1) the incorporation of plant proteinase inhibitors into artificial diets for laboratory-grown pests, (2) feeding studies using plant tissue with or without proteinase inhibitory activity, and (3) the use of plants that have been transformed with a gene for proteinase inhibitor.

The potency of specific proteinase inhibitors is dependent on the presence of (1) susceptible proteinases in the target organism, and (2) other dietary factors (i.e. protein quality, polyphenyloxidase activity). The susceptibility of a particular proteinase protein is dependent upon (1) its type of proteinase activity (e.g. trypsin, chymotrypsin, carboxypeptidase), and (2) the structural configuration of its active site (the site of interaction between the enzyme and its inhibitor); the stronger the interaction between the inhibitor and enzyme, the more effective the inhibitor.

Thus, although proteinase inhibitors in general may contribute to the defense of plants against attack by invading pests, the efficacy of specific inhibitors from individual species of plants is dependent upon (1) the unique structure of the plant proteinase inhibitor, and (2) the susceptibility of the proteinase in the target organism. It appears that each plant species produces a proteinase

inhibitor having a unique structure, and that while the proteinase inhibitor produced by one plant type may have a protective effect upon the pests which feed upon that type, the inhibitor may have little or no protective effect against the herbivorous pests feeding upon a second plant type.

I have recently reported that cabbage has significantly higher levels of trypsin inhibitory activity than many other crucifers [Tryptic inhibitory activity in wild and cultivated crucifers. *Phytochemistry* , 28:755 (1989)], and that larval *Trichoplusia ni* and *Pieris rapae* both rely upon trypsin and chymotrypsin for digestion of protein [Characterization and ecological implications of midgut proteolytic activity in larval *Pieris rapae* and *Trichoplusia ni*. *Journal of Chemical Ecology* 15:2101 (1989)]. Based upon these findings, the initial scientific direction of the present invention was to isolate, purify, characterize, and determine the effect the proteinase inhibitory agents of cabbage would have have on *T. ni* and *P. rapae* larvae.

The following disclosure and examples are provided to allow one to receive a more complete understanding of the present invention. These examples are not intended nor provided to limit the scope of the present invention in any manner, and it would be improper for one to interpret them as doing so.

EXAMPLE I

Seeds for the cabbage cultivar Superpack™ were germinated in Cornell Mix™ in 2 gallon plastic pots, The seedlings were thinned to one plant per pot, and maintained under greenhouse conditions under 1 kw metal halide lamps at 28°C. The plants were watered daily and fertilized once a week with a water-soluble nutrient (16-32-16) mix.

Two differing whole plant bioassays were conducted: (1) each plant was placed in a large cage and larvae of *Trichoplusia ni* and *Pieris rapae* were allowed to move about freely on the entire plant; and (2) larvae were confined to a specific leaf in a 2-inch diameter cage which was moved when the foliage within the cage was consumed. For each type of bioassay, both young (10 to 12 true leaves) and mature (at least 3-inch diameter plants with 9 to 11 frame leaves) plants were used. In addition, a control plant without larvae infestation was placed in the same conditions as the treated plants and chemically analyzed at the end of the experiment.

For the first bioassay, 40 *Trichoplusia ni* eggs or 100 *Pieris rapae* eggs were applied to each of two mature plants per insect species. The larger number of *P. rapae* eggs placed on each plant was to overcome the greater loss of larval *P. rapae* during the experiments, possibly because of the influence of crowding. Each of the plants was individually housed in an insect proof cage to prevent the migration of insects to neighboring plants. The entire

experiment was terminated when a single type of foliage (e.g. the young leaf tissue on young plants) was substantially consumed. All the larvae were weighed, and the plants were chemically analyzed for tryptic inhibitory activity, chymotryptic inhibitory activity, total protein, and total glucosinolates.

For the experiments in which the larvae were confined to a specific leaf on a plant, 50 *P. rapae* eggs were placed inside the small cage (with a recovery of 10 to 20 larvae) and, as they grew, the larvae were separated into 3 to 5 larvae per cage. Thirty eggs for *T. ni* were placed inside a small cage and separated into 3 to 5 larvae per cage as the larvae grew. The cages were placed on an expanding leaf (leaf numbers 3 to 5) based on the designation of leaf number 1 as the youngest expanding leaf and each successive number indicating the next oldest leaf on the plant) on each young plant. For each mature plant, one cage was placed on a young expanding leaf (leaf numbers 2 to 4) and one cage was placed on a mature leaf (leaf numbers 8 to 10). The entire experiment was terminated when a single type of foliage (e. g. the young leaf tissue on a young plant) was substantially consumed. All the larvae were weighed, and the plants were chemically analyzed for tryptic inhibitory activity, chymotryptic inhibitory activity, total protein, and total glucosinolates.

With regard to total plant protein bioassay, the total protein fractions were isolated from three different types of cabbage

foliage: (1) young leaves (leaf numbers 1 to 4) from mature plants, (2) mature leaves from mature plants, or (3) leaves from young plants. The foliage (700 grams) was homogenized in 500 ml of 0.01 M sodium citrate, 1 M potassium chloride, pH 4.5 buffer. The supernatant was retained and the homogenate was pressed through two layers of cheesecloth, and the liquid was collected, on ice. The leaf tissue was homogenized a second time in 500 ml of buffer, pressed through the cheesecloth, and the collected liquid was pooled with the first supernatant. The liquid was centrifuged at 4200 x g for 15 min at 4°C. The supernatant was collected, and ammonium sulfate was added to 70% saturation. The solution was stored overnight at 4°C, and centrifuged at 4200 x g for 15 min at 4°C. The pellet was resuspended in a small volume of distilled water, and subsequently dialyzed (12,000 to 14,000 MWCO) against distilled water. The dialysate was centrifuged at 4200 x g for 15 min at 4°C, and the supernatant was lyophilized. The lyophilized powder was chemically analyzed for tryptic inhibitory activity, chymotryptic inhibitory activity and total protein. The powder remaining after analysis was used to prepare 600 ml of wheat germ based diet for the determination of the effect of this foliar total protein on larval growth and development. The diet used in these tests was the same as that used for mass rearing of larval *P. rapae* except that the concentration of casein in the diet was reduced from 3.2% to 1.6 % (weight/volume). The bioassays for both insect species included 2

treatments, 3 cups per treatment, 30 eggs per cup. Each bioassay was replicated. The larvae were provided with diet, *ad libitum* from neonate until the controls reached the ultimate instar at which point tall the larvae were weighed.

With regard to chemical analyses, a standard spectrophotometric assay was used to determine the presence of tryptic inhibitory activity in the cabbage foliage. Bovine trypsin (0,1 mg/ml, 1 mM HCl) was mixed (1:1 volume/volume) with cabbage leaf juice acquired by grinding the foliage with a mortar and pestle, centrifuging the tissue, collecting the supernatant, and diluting the supernatant to 0.1x with 1 mM HCl. The resulting material was then incubated at room temperature for 10 min. Then 100 µl of the mixture was added to 2.9 ml of buffer (0.05 M Tris, pH 8.0) containing 1.04 M p-toluene-sulphonyl-L-arginine methyl ester. Tryptic activity was monitored at 247 nm for 3 min utilizing a 50 µl aliquot of trypsin to determine uninhibited tryptic activity.

Chymotryptic inhibitory activity was determined by mixing (1:1 volume/volume) the diluted leaf juice as prepared above with TLCK-treated bovine chymotrypsin (0,1 mg/ml 1 mM HCl) for 10 min at room temperature. 100 µl of the mixture was then added to 2.9 ml substrate (1 mM benzoyl-L-tyrosine ethyl ester in 50% MeOH, mixed 1:1 with 0.05 M Tris buffer at pH 8.0), and monitored spectrophometrically at 256 nm for 3 min. A 50 µl aliquot of

chymotrypsin was used to determine uninhibited chymotryptic activity.

To quantify glucosinolates in cabbage foliage, leaves were ground with a mortar and pestle, and the fluid was applied to a 1 ml DEAE Sephadex A25 column, The column was washed with distilled water followed by duplicate washings with 0.5 ml 0.02 M pyridine/acetic acid buffer. Myrosinase (250 µl of 25 mg/ml pyridine buffer) was applied to the column and allowed to incubate at room temperature for 2 hours. The column was then eluted with distilled water, collecting 1.25 ml of eluant. Three 250 µl aliquots of the eluant were assayed for glucose with sinigrin used as the standard.

With regard to total protein concentration, bicinchoninic acid reagent was used with purified protein from cabbage foliage being used as the standard.

Following these experimental procedures, the larvae that hatched on plants in the large cages were allowed to select their feeding sites. Larval *T. ni* fed on the underside of the oldest leaves of both the young and mature plants, although they would move up the plant to the fully expanded mature leaves as the oldest leaf tissue was consumed. The preferred feeding site for larval *P. rapae* was the youngest tissue on the plant. They consumed the apical meristem of the plant first, then moved downward feeding on the non-vascular tissue at the base of the youngest leaves. They generally did not feed on the fully expanded or oldest leaves of either the young or mature plant.

When larval *T. ni* were free to select their feeding sites (thus feeding on the oldest leaves on the plant), there was no significant difference in growth between larval *T. ni* feeding on the young or mature plants ($p = 0.460$, $n = 220$). The chemical analyses of the leaf tissue indicated that there was no significant difference in the tryptic inhibitory activity ($p = 0.383$, $n = 35$),chymotryptic inhibitory activity ($p = 0.389$, $n = 35$), total protein ($p = 0.563$, $n = 35$), or total glucosinolate $p > 0.05$, $n = 35$) in the oldest foliage from the young and mature plants. Larval *P. rapae* that fed on the youngest foliage on mature plants were significantly smaller than larvae that fed on the youngest foliage on young plants ($p < 0.001$, $n = 87$). A comparison of the chemical composition of plants indicated that the tryptic and chymotryptic inhibitory activity in the young

foliage on the mature plant was significantly higher than that on the young plant ($p < 0.001$, $n = 35$ for both trypsin and chymotrypsin).

Larval *T. ni* that were confined to feeding on the oldest leaves on the mature plant were significantly larger than the larvae feeding on the young leaves on the young plant ($p < 0.001$, $n = 45$). The larvae restricted to feeding on the young leaves on the mature plant attempted to feed, but there was 100% mortality of the larvae in the second instar. Examination of the chemical analyses of the plant foliage indicated that there was a significant inverse correlation between larval growth and the level of tryptic or chymotryptic inhibitory activity. The tryptic and chymotryptic inhibitory activity was significantly highest in the young leaves on the mature plant ($p < 0.001$, $n = 35$), at an intermediate level in the young leaves on the young plant ($p < 0.001$, $n = 35$), and at the significantly lowest level in the old leaves on the mature plant ($p < 0.001$, $n = 35$).

With the findings of Example I, the effect of cabbage proteinase inhibitor on the growth and development of larval *T. ni* and *P. rapae* were examined in greater detail according to the following example.

EXAMPLE II

Proteinase inhibitors were extracted from cabbage by homogenizing the foliage in 0.01 M sodium citrate I M potassium chloride buffer at pH 4.5, centrifuging the homogenate at 4200 x g for 10 min at 4°C, and collecting the supernatant. The supernatant was incubated for 10 min at 70°C, cooled on ice, and centrifuged at 6000 x g for 60 min. The supernatant was adjusted to pH 8.0, ammonium sulfate was added to 70% saturation, and the resulting solution was stored overnight at 4°C. The solution was then centrifuged at 6000 x g, and the pellet was resuspended in distilled water, dialyzed (MWCO 12,000 to 14,000) against water to remove the salt. The dialysate was centrifuged at 6000 x g for 20 min, and the supernatant lyophilized and labeled as "semi-pure proteinase inhibitor".

The semi-pure proteinase inhibitor was purified in a two-step process. First, 100 mg of semi-pure material was applied to a Sephadex-G75 column (2.2 x 50 cm) previously equilibrated and eluted with 0.05 M Tris pH 9.0 buffer, and monitored at 280 nm. Protein fractions with tryptic inhibitory activity were pooled, dialyzed against distilled water, lyophilized, and labeled "Sephadex-purified proteinase inhibitor". The second step in the purification process involved trypsin-bound cyanogen bromide activated Sepharose 4B affinity chromatography. The proteinase inhibitor was

applied to the affinity column (1.5 x 33 cm) in 0.01 M Tris 0.1 M calcium chloride buffer at pH 8.1. The column was washed with the buffer until the optical density (at 280 nm) approached zero. The trypsin inhibitor was then eluted with 8 M urea at pH 3.0, dialyzed against distilled water, lyophilized, and labeled "affinity-purified proteinase inhibitor".

To determine the purity of the proteinase inhibitors prepared as above, a sample (4 mg/ml) of each preparation was applied to a vertical, discontinuous, non-denaturing 12.5% polyacrylamide gel with a 4% stack. Following electrophoresis, the gel was stained with 0.1% Coomassie brilliant blue R in 20% MeOH, 30% acetic acid, and destained with 30% MeOH, 10% acetic acid.

The presence of tryptic and chymotryptic inhibitory activities in the sample preparations was determined using procedures modified slightly from those described in Example 1.

The presence of tryptic inhibitory activity in the cabbage extracts and purification fractions was determined by mixing bovine trypsin (0.1 mg/ml, 1 mM HCl) 1:1 (volume/volume) with the plant extract (2 mg/ml, 1 mM HCl), incubating the resulting mixture for 10 min at room temperature, and then adding 2.9 ml of buffer (0.05 M Tris at pH 8.0 and containing 1.04 M p-toluene-sulphonyl-L-arginine) to 100 µl of the mixture. Tryptic activity was monitored at 247 nm for 3 min. A 50 µl aliquot of trypsin was used to determine uninhibited tryptic activity.

Chymotryptic inhibitory activity was determined by mixing (1:1 volume/volume) the test solution (2 mg/ml, 1 mM HCl) for 10 min at room temperature. 100 µl of the mixture was then added to 2.9 ml of substrate (1 mM benzoyl-L-tyrosine ethyl ester in 50% MeOH, mixed 1:1 with 0.05 M Tris pH 8.0 buffer), and monitored spectrophotometrically at 256 nm for 3 min. A 50 µl aliquot of chymotrypsin was used to determine uninhibited activity.

To determine the effect of cabbage proteinase inhibitors on larval growth and development, larvae were reared on a wheat germ-based diet that was supplemented with plant proteinase inhibitors. The basic diet was the same as that used in Example I. Initial experiments were performed using semi-purified proteinase inhibitor. Soybean trypsin inhibitor was used as a standard for comparison. Each bioassay included 5 treatments, 3 cups/treatment, 30 eggs/ cup. To ensure the results for the semi-purified proteinase inhibitor were due to the inhibitor ans not a contaminant in the preparation, the bioassay was repeated using the Sephadex-purified proteinase inhibitor. As a final confirmation of the toxicity of cabbage trypsin inhibitor to larval *T. ni* and *P. rapae*, a bioassay was also performed using affinity-purified trypsin inhibitor. For all tests, the larvae were started on the diets as neonates, and were maintained on the diets until the controls reached the ultimate instar. All larvae were then weighed. The percent pupation and

adult emergence was based on the total number of larvae weighed for each diet.

Following the incorporation of the different proteinase inhibitor preparations into the artificial diet, the soybean trypsin inhibitor had no significant effect on the growth of larval *T. ni* ($p = 0.225$, $n = 825$) or *P. rapae* ($p = 0.206$, $n = 991$) even at a concentration of 0.5% (weight/volume) in the diet. This evidences an adaption of each plant species to produce its own proteinase inhibitor specific for the trypsin and chymotrypsin inhibition required to protect itself against the species of larvae which utilizes the host plant as a source of food. Growth of larval *T. ni* and *P. rapae*, however, was significantly reduced by dietary supplementation with semi-purified ($p < 0.001$, $n = 1252$ for *T. ni*; $p < 0.001$, $n = 1557$ for *P. rapae*); Sephadex-purified ($p < 0.001$, $n = 264$ for *T. ni*; $p < 0.001$, $n = 270$ for P. rapae); and affinity-purified proteinase inhibitory factors. In addition, the dietary concentration of the proteinase inhibitor acted as a predictor of larval growth.

The relative proportion of larvae that pupated was also found to be significantly influenced by the presence of dietary cabbage inhibitor. In addition, pupal deformities were also seen as being common for larvae feeding on diets containing proteinase inhibitor. The dietary concentration of proteinase inhibitor also acted as a predictor of percent pupation of larval *T. ni* and *P. rapae*.

Although many plant proteinase inhibitors have been shown to reduce the growth and development of insects, the remarkable feature about the results from Examples I and II is the level of

dietary cabbage proteinase inhibitor required to significantly reduce larval growth and development. With cabbage proteinase inhibitor, a dietary concentration of 0.1% (1 mg/ml) will reduce larval growth as much as 66%, reduce pupation by 93%, and reduce adult emergence by 60%. Thus, these proteinase inhibitors have a sufficient detrimental effect on the growth and development of larval pests to make them potentially valuable as insecticides and larvacides for commercial use.

Although proteinase inhibitors are believed to have a role in the defense of plants against attack by herbivorous insects, detailed characterization of specific proteinase inhibitors is necessary to determine there effectiveness against specific attacking organisms. The effectiveness of a proteinase inhibitor as a defensive agent is directly related to its stability (both pH and temperature), specificity, binding constant, and concentration. Such information has been reported for proteinase inhibitors from legumes, solanum, and grains. However, before the making of the present invention, nothing was known about the chemical structure and biological activity of the proteinases from Cabbage. Accordingly, the following example was conducted with the specific purpose of determining the chemical structure and characteristics of the protein inhibitors isolated and tested in Examples I and II.

EXAMPLE III

Proteinase inhibitor was extracted from cabbage by homogenizing 500 grams of fresh foliage in 900 ml of 0.01 M sodium citrate, 1 M KCl, pH 4.5 buffer. The homogenate was squeezed through a double layer of cheesecloth, and the liquid was centrifuged at 4200 x g for 10 min at 4°C. The supernatant was incubated for 10 min at 70°C, then cooled on ice for 20 min and centrifuged at 6000 x g for 60 min. The supernatant was adjusted to pH 8.0 with NaOH, and the protein was precipitated with ammonium sulfate (70% saturation) at 4°C overnight. The sample was centrifuged at 6000 x g for 20 min, and the pellet was resuspended in dwater, and dialyzed (MWCO 12,000 to 14,000) against dwater to remove the salt. The dialysate was then lyophilized and labeled as "semi-purified proteinase inhibitor".

The semi-purified proteinase inhibitor was purified by column chromatography. A 200 mg sample of the inhibitor was applied to an anion exchange 4.5 x 9 cm column with a DEAE-25A Sephadex bed, and the column was washed with 10 column volumes of 0.05 M Tris pH 9.0 buffer (until the optical density at 280 nm returned to zero). This removed most of the chlorophilic material from the sample. Then, collecting 9 ml fractions, the proteinase inhibitor material was eluted with 0.2 M Tris pH 8.5 buffer (an additional protein beak was eluted with 0.5 M Tris, pH 7.8 buffer but showed no proteinase

inhibitory activity). The proteinase inhibitory active fractions from the column were applied to an affinity column of trypsin bound to cyanogen bromide activated Sepharose 4B at 6°C. The column was washed with 10 column volumes of 0.01 M Tris 0.1 calcium chloride pH 8.1 buffer (until O.D. drops equaled 300 to 500 ml). The proteinase inhibitor was eluted with 8 M urea at pH 3.0 (until O.D. dropped <100 ml), and the fractions from the entire protein peak (280 nm) were pooled. The protein peak (50 to 60 ml) was dialyzed against dwater (16 to 20 liters) overnight, concentrated to 2 ml, and analyzed for tryptic and chymotryptic inhibitory activity.

The thermal stability of the proteinase inhibitory activity was tested by incubating aliquots of a solution of semi-purified proteinase inhibitor (2 mg/ml, 1 mM HCl) at designated temperatures for specific lengths of time. Each solution was then mixed (1:1 volume/volume) with bovine trypsin or alpha-chymotrypsin (0.1 mg/ml, 1 mM HCl), incubated for 10 min at room temperature, and tested for enzyme activity.

To determine the purity of the affinity purified proteinase inhibitor, 75 µl of concentrate was applied to a vertical, discontinuous, non-denaturing, 12.5% polyacrylamide gel with a 4% stack (using 0.025 M Tris 0.192 M glycine pH 8.3 buffer as the reservoir buffer). Following electrophoresis, the gel was stained with Coomassie brilliant blue R (20% MeOH, 30% acetic acid,).1% Coomassie) and de-stained with 30% MeOH, 10% acetic acid to detect

all protein bands. To determine those protein bands with tryptic inhibitory activity, a modification of the method from Filho and Moreira (1978) was used. The native gel was washed 3 times with 30% MeOH, 20% acetic acid to fix the protein, and then rinsed with distilled water 2 times. The gel was equilabrated, overnight, in 0.1 M sodium phosphate buffer at pH 7.8, and then incubated in a trypsin solution (0.1 ml trypsin/ml 0.1 M phosphate buffer at pH 7.8) for 30 min at 37°C. The gel was rinsed twice with distilled water, then covered with a freshly prepared solution of 2.5 mg acetyl-phenylanine-ß-naphthyl-ester in 1 ml dimethylformamide plus 9 ml of 0.55 mg/ml tetrazotized O-dianisidine in 0.1 M phosphate buffer at pH 7.8. The gel was incubated in the acetyl-phenylalanine-ß-naphthyl-ester solution for 30 min at 37°C, then rinsed with distilled water. Clear bands in the final gel indicated the presence of trypsin inhibitor.

The molecular weights of the proteins with proteinase inhibitory activity were determined on PAGE-SDS. A 1.5 mm discontinuous polyacrylamide gel consisting of a 15% acrylamide-0.34% bis-acrylamide separating gel and a 4% acrylamide-0.1% bis-acrylamide stacking gel were used. A 75 µl sample plus molecular weight markers with molecular weight range of 14,000 to 70,000 were employed in the PAGE-SDS method.

The isoelectric point for each trypsin inhibitor was also determined using conventional methods known in the art.

A standard spectrophotometric assay was used to determine the presence of tryptic inhibitory activity in the cabbage extracts and purification fractions. Bovine trypsin (0.1 mg/ml 1 mM HCl) was mixed (1:1 volume/volume) with the plant extract, and then incubated at room temperature for 10 min. 100 µl of the mixture was then added to 2.9 ml of buffer (0.05 M Tris at pH 8.0) containing 1.04 M p-toluene-sulphonyl-L-arginine methyl ester. Tryptic activity was monitored at 247 nm for 3 min.

Chymotryptic inhibitory activity was determined by mixing the test solution (1:1 volume/volume) with TLCK-treated bovine chymotrypsin (0.1 mg/ml 1 mM HCl) for 10 min at room temperature. 100 µl of the mixture was then added to 2.9 ml substrate (1 mM benzoyl-L-tyrosine ethyl ester in 50% MeOH, mixed 1:1 with 0.05 M Tris buffer at pH 8.0), and monitored at 256 nm for 3 min.

Following the procedures outlined in example III, both tryptic and chymotryptic inhibitory activity were detected in the crude extract of homogenized foliage from mature plants. However, with each purification step, the ration of tryptic to chymotryptic inhibitory activity increased (i.e., the level of tryptic activity increased) as depicted in the following table:

TABLE I

Treatment	Tryptic:Chymotryptic Inhibitory ratio
Crude leaf juice	2:1
Semi-purified proteinase inhibitor	2:1
DEAE-purified proteinase inhibitor	10:1
Affinity-purified proteinase inhibitor	70:1

In addition, both types of inhibitory activity were very stable at high temperatures, in an acidic environment, and were found to be significantly reduced only when lyophilized.

After the initial isolation and removal of thermally unstable proteins, DEAE chromatography removed the majority of the chlorophyll and a significant proportion of contaminating protein. Affinity chromatography was used to purify the trypsin inhibitors. Approximately 5 to 6 hrs were required to apply the 300 to 400 of DEAE-purified trypsin inhibitor samples to the affinity column. The column was then washed with buffer until the column eluates no

longer contained material absorbing at 280 nm. Subsequent elution with <100 ml of 8 M urea, pH 3.0, eluted a single peak of 280 nm absorbing material which contained tryptic inhibitory activity. Washing the column with 1 mM HCl, 0.1 M $CaCl_2$ eluted a second protein peak that had no tryptic inhibitory activity.

Three protein bands for the affinity purified trypsin inhibitory agents according to the present invention were detectable when examined in a non-denaturing, discontinuous 12.5% polyacrylamide gel. The two major bands represented approximately 45% each of the total protein, while the minor band represented the remainder. All three bands were found to have tryptic inhibitory activity. The molecular weights of these three proteins was found to be 23,300, 22,250, and 18,370 daltons; the isoelectric points for the three proteins were 5.05, 5.13, and 5.99.

The proteinase inhibitors of the present invention have a number of potential uses as insecticides for herbivorous pests which attack cabbage plants. The proteinases inhibitors having the molecular weights and isoelectric points isolated from cabbage tissues may be utilized in sprays or powders (in combination with conventional insecticides, surfactants, buffers, fillers, binding agents and other materials commonly compounded with insecticidal sprays and powders, including materials to inhibit the degrading of the proteinase inhibitor by environmental means such as sunlight or bacteria after application) which are meant to be applied to the

growing cabbage plant as a means to control and prevent *T. ni* and *P. rapae* larval infestation of the plant. Of course, rather than physical application of proteinase inhibitors to growing cabbage plants, it is also within the intention of the present invention to encompass the use of such inhibitory agents through biotechnical means. For example, using conventional technology, the amino acid sequence of the inhibitory agents may be determined; once known, the genetic sequence necessary to produce the inhibitory agent by the plant may be determined; and this sequence may then be incorporated, along with an appropriate genetic promoter to initiate expression of the sequence if necessary, within the genome of the plant, thus giving the plant the innate ability to produce sufficient amounts of the inhibitory agent to protect all its plant tissues from attack by susceptible pests.

Thus, while I have illustrated and described the preferred embodiment of my invention, it is to be understood that this invention is capable of variation and modification, and I therefore do not wish or intend to be limited to the precise terms set forth, but desire and intend to avail myself of such changes and alterations which may be made for adapting the invention of the present invention to various usages and conditions. Accordingly, such changes and alterations are properly intended to be within the full purview of the the following claims. The terms and expressions which have been employed in the foregoing specification are used

therein as terms of description and not as terms of limitation, and thus there is no intention, in the use of such terms and expressions, of excluding equivalents of the features shown and described or portions thereof, it being recognized that the scope of the invention is defined and limited only by the claims which follow.

Having thus described my invention and the manner and process for making and using it in such full, clear, concise, and exact terms so as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected to make and use the same,

I CLAIM:

1. A proteinase inhibitory agent which is a peptide having a molecular weight of from about 18,370 to about 23,300 daltons, an isoelectric point of from about 5.05 to about 5.99, and which is inhibitory to trypsin and chymotrypsin produced in *Trichoplusia ni* and *Pieris rapae* larvae.

2. A proteinase inhibitory agent according to claim 1 wherein the molecular weight is about 18,370 daltons and the isoelectric point is about 5.99.

3. A proteinase inhibitory agent according to claim 1 wherein the molecular weight is about 23,300 daltons and the isoelectric point is about 5.05.

4. A proteinase inhibitory agent according to claim 1 wherein the molecular weight is about 22,250 daltons and the isoelectric point is about 5.13.

5. A method for inhibiting the growth and development of *Trichoplusia ni* and *Pieris rapae* larvae which comprises providing said larvae a proteinase inhibitory agent having a molecular weight of from about 18, 370 to about 23,300 daltons and an isoelectric point of from about 5.99 to about 5.05, and allowing said larvae to ingest the agent.

6. A method according to claim 5 wherein said agent is provided by applying said agent to the foliage of a cabbage plant.

7. A method according to claim 6 wherein said agent is in solution and applied as a spray.

8. A method according to claim 6 wherein said agent is a solid and applied as a powder.

9. A method for providing a cabbage plant with the ability to protect itself against damage caused by the infestation of larvae of *Trichoplusia ni* or *Pieris rapae* which comprises inserting the genetic sequence for the production of a proteinase inhibitory peptide specific for a proteinase found in the body of said larvae into the genome of said plant, providing means to allow said transformed genome to initiate expression of said sequence, and initiating the expression of said inhibitory peptide.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 15/10; A01N 65/00; C12N 15/82
U.S.Cl.: 530/370, 379; 514/2; 435/69.2

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.Cl.	530/370, 379; 514/2; 435/69.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Searched DIALOG Files 5, 10, 35, 155, WPI, 340, 357 and 399 and APS for protease inhibitors of similar MW and pI and the use of inhibitors to control insects.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	U.S.A. 4,640,836 (Boulter et al.). 03 February 1987. see the abstract and columns 2. 8 and 9.	9
Y	EP.A. 0.339.009 (Fuche et al.). 25 October 1989. see whole publication. especially pages 2 and 3.	9
Y	Insect Biochemistry. Vol. 11, No. 5. issued 1981. Prichett et al.. "Proteolytic activity in the digestive fluid of larvae of Trichoplusia ni". pages 523-526. See page 523.	9
Y	Nature. Vol. 330. issued 12 November 1987. Hilder et al.. "A novel mechanism of insect resistance engineered into tobacco", pages 160-163. See whole publication.	9

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 February 1991	04APR 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Keith C. Furman

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Chemical Abstract. Vol. 13. issued 1982. Fukuzawa et al.. "Purification and properties of trypsin inhibitor from cabbage seed (B. Oleracea L. var. Capitata)". see Chemical Abstracts No. 99:118269s of Seitoku Eiyo Tanki Daigaku.

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

European Patent Office

SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number

EP 91 90 2042

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
D,A	CHEMICAL ABSTRACTS, vol. 110, no. 17, 24 April 1989, Columbus, Ohio, US; abstract no. 151365, R. M. ROXANNE ET AL.: 'Tryptic inhibitory activity in wild and cultivated crucifers' page 440 ; column L ; * abstract * & PHYTOCHEMISTRY vol. 28, no. 3, 1989, NEW YORK, US pages 755 - 758; ---	1-4	C07K15/10 A01N65/00 C12N15/82
A	BIOLOGICAL ABSTRACTS. - MICROFILMS. vol. 90, 1990, PHILADELPHIA, PA US; ABSTR. 139179 A. WILIMOWSKA-PELC: 'Trypsin inhibitors in turnip (Brassica rapa L.) seeds' & ACTA SOC. BOT. POL. vol. 58, no. 4, 1989, WARSAW, PO pages 563 - 574; ---	1-4	
A	BIOLOGICAL ABSTRACTS. - MICROFILMS. vol. 91, 1991, PHILADELPHIA, PA US; ABSTR. 134724 I. B. SVENDSEN ET AL.: 'Isolation and characterization of a trypsin inhibitor from the seeds of kohlrabi (brassica napus var. rapifera)' & CARLSBERG RES. COMMUN. vol. 54, no. 6, 1989, COPENHAGEN, DK pages 231 - 240; -----	1-4	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C07K A01N C12N

The supplementary search report has been drawn up for the claims attached hereto.

Place of search	Date of completion of the search	Examiner
THE HAGUE	02 JULY 1992	THIELE U.H.-C.H.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0400)

I CLAIM:

1. A proteinase inhibitory agent which is a peptide having a molecular weight of from about 18,370 to about 23,300 daltons, an isoelectric point of from about 5.05 to about 5.99, and which is inhibitory to trypsin and chymotrypsin produced in *Trichoplusia ni* and *Pieris rapae* larvae.

2. A proteinase inhibitory agent according to claim 1 wherein the molecular weight is about 18,370 daltons and the isoelectric point is about 5.99.

3. A proteinase inhibitory agent according to claim 1 wherein the molecular weight is about 23,300 daltons and the isoelectric point is about 5.05.

4. A proteinase inhibitory agent according to claim 1 wherein the molecular weight is about 22,250 daltons and the isoelectric point is about 5.13.

5. A method for inhibiting the growth and development of *Trichoplusia ni* and *Pieris rapae* larvae which comprises providing said larvae a proteinase inhibitory agent having a molecular weight of from about 18, 370 to about 23,300 daltons and an isoelectric point of from about 5.99 to about 5.05, and allowing said larvae to ingest the agent.

6. A method according to claim 5 wherein said agent is provided by applying said agent to the foliage of a cabbage plant.

110: 151364n The carbon–sulfur lyases of higher plants. Part 5. Lack of homology between the alliin lyases of garlic and onion. Nock, Linda P.; Mazelis, Mendel (Dep. Food Sci. Technol., Univ. California, Davis, CA 95616 USA). *Phytochemistry* 1989, 28(3), 729–31 (Eng). The genus *Allium* contains a no. of important food plants whose characteristic flavors and odors are produced by the action of the enzyme alliin lyase (EC 4.4.1.4) on endogenous non–protein *S*–contg. amino acids. Previous studies had shown great differences in the purified enzyme from onion (*A. cepa*) and garlic (*A. sativum*). The degree of homol. of the enzyme protein from these two sources was examd. based on its immunol. cross–reaction with polyclonal antibodies produced by homogeneous enzyme from each species. The results show no cross–reaction of the garlic enzyme antibodies with the onion enzyme, and an inconsistent amt. of cross–reaction of the garlic enzyme with onion antibodies. In addn. *N*–terminal amino acid anal. shows a large difference between the purified enzyme from these two sources.

110: 151365p Tryptic inhibitory activity in wild and cultivated crucifers. Broadway, Roxanne M. (Dep. Entomol., New York State Agric. Exp. Stn., Geneva, NY 14456 USA). *Phytochemistry* 1989, 28(3), 755–8 (Eng). Tryptic inhibitory activity was detected in all species and cultivars of crucifers examd. When comparing the 3 groups of crucifers tested [(i) foliage of cultivated crucifers, (ii) foliage of wild crucifers and (iii) storage organs of cultivated crucifers], the foliage of cultivated crucifers contained significantly higher levels of tryptic inhibitory activity than the other two groups.

110: 151366q Electrophoresis and electrofocusing of phytochrome from etiolated Avena sativa L. Schendel, Rudolf; Ruediger, Wolfhart (Bot. Inst., Univ. Muenchen, Munich. Fed. Rep. Ger.). *Z. Naturforsch., C: Biosci.* 1989, 44(1–2), 12–18 (Eng). Phytochrome from etiolated oat seedlings (*A. sativa*) was investigated by native gel electrophoresis and by isoelec. focusing. At pH 8.8 the far–red (Pfr) form migrated faster than the red (Pr) form in electrophoresis. A difference was assumed in the surface charge rather than in shape for the phytochrome forms. This assumption was confirmed by isoelec. focusing which clearly showed relatively more neg. charge in the Pfr form than in the Pr form. The role of the peptide region from residue 323 to 360 is discussed in this connection. It carries 9 neg. charged residues, it is exposed only in the Pfr form and it has already been described as a signal region for rapid protein degrdn. The expts. on electrofocusing revealed a microheterogeneity of phytochrome which was present in the native state as well as in the completely unfolded state. The most probable reason could be either posttranslational modification or genetic polymorphism of phytochrome in oat.

110: 151367r Plant cells contain calsequestrin. Krause, Karl Heinz; Chou, Mei; Thomas, Mitchell A.; Sjolund, Richard D.; Campbell, Kevin P. (Coll. Med., Univ. Iowa, Iowa City, IA 52242 USA). *J. Biol. Chem.* 1989, 264(8), 4269–72 (Eng). Calsequestrin is a high capacity low affinity Ca^{2+}–binding protein thought to be essential for the function of the intracellular rapid releasable Ca^{2+} pool of a variety of animal cells. Cultured *Streptanthus tortuosus* cells and spinach leaves contain a form of calsequestrin. In subcellular fractions of *S. tortuosus* cells, Stains–all staining reveals a metachromatically blue–staining 56–kilodalton protein enriched in the microsomal fraction. This protein shares several biochem. characteristics with animal calsequestrin: (*1*) it changes its apparent mol. wt. with the pH; (2) it is able to bind $^{45}Ca^{2+}$ on nitrocellulose transfers; and (*3*) it is recognized by antibodies against canine cardiac calsequestrin. Calsequestrin was also identified in spinach leaves using a direct extn. procedure that was developed for muscle calsequestrin. Thus, results demonstrate that plant cells contain calsequestrin within a subcellular membrane fraction. These results also suggest that calsequestrin is an ubiquitous protein rather than being limited only to animal cells.

110: 151368s Characterization of the isolated calcium–binding vesicles from the green alga Mougeotia scalaris, and their relevance to chloroplast movement. Grolig, Franz; Wagner, Gottfried (Bot. Inst. I, Justus–Liebig–Univ., D–6300 Giessen. Fed. Rep. Ger.). *Planta* 1989, 177(2), 169–77 (Eng). The calcium–binding vesicles from the green alga *M. scalaris* were isolated and characterized after staining in vivo by neutral red or rhodamine B. They had a protonated group with a pK_a = 9.9, typifying phenolic hydroxyl groups; upon titrn., both, phenolic compd.(s) and vital dye were concomitantly released from the vesicular matrix. A shift in peak absorbance from 450 nm to 540 nm of the vitally stained vesicles indicated that the neutral form of neutral red was bound to the vesicular matrix as an intermediate form, stabilized via intermol. H bonds to the phenolic compd.(s). Up to $8.5.10^9$ dye mols. were adsorbed to a mean–size vesicle. Anal. of Langmuir adsorption isotherms indicated that there were two binding sites each for both neutral red and rhodamine B. These isolated vesicles were devoid of Ca, probably because vesicular Ca, bound to the vesicle matrix, was displaced upon dye binding. Dye adsorption to the vesicles in vivo results in substantial inhibition of the reorientational movement of the *Mougeotia* chloroplast and is explained by dye–mediated disorder of the cellular Ca homoeostasis.

110: 151369t A stable blue–light–derived signal modulates ultraviolet–light–induced activation of the chalcone–synthase gene in cultured parsley cells. Ohl, S.; Hahlbrock, K.; Schaefer, E. (Inst. Biol. II/Bot., Univ. Freiburg, D–7800 Freiburg/Br., Fed. Rep. Ger.). *Planta* 1989, 177(2), 228–36 (Eng). Run–off transcription assays were used to demonstrate that both the UV–B and blue–light receptors control transcription rates for chalcone–synthase mRNA in the course of light–induced flavonoid synthesis in parsley (*Petroselinum crispum* cell–suspension cultures. Blue and red light alone, presumably acting via a blue–light receptor and active phytochrome (far–red absorbing form) resp., can induce accumulation of chalcone–synthase mRNA. The extent of the response is however considerably smaller than that obtained when these wavebands are applied in combination with UV light. A preirradn. with blue light strongly increases the response to a subsequent UV pulse and this modulating effect of blue light is abolished by a UV induction but can be reestablished by a second irradn. with blue light.

110: 151370m Microtubule–binding proteins from carrot. I. Initial characterization and microtubule bundling. Cyr, Richard J.; Palevitz, Barry A. (Dep. Bot., Univ. Georgia, Athens, GA 30602 USA). *Planta* 1989, 177(2), 245–60 (Eng). From suspension–cultured cells of carrot (*Daucus carota*), non–tubulin proteins were isolated which bind to and affect microtubules (MTs) in vitro. These proteins were isolated using taxol–stabilized neuronal MTs as an affinity substrate. They cause MT bundling at substoichiometric concns., support the assembly of tubulin in vitro, and at low concns., decorate single MTs in a periodic fashion. The bundled MTs formed in vitro share similarities with those seen in situ in a variety of plant cells, including a center–center spacing of 34 nm, cold stability, resistance to anti–microtubule drugs, and sensitivity to Ca. The bundling activity is specific: other cationic proteins, as well as poly–L–lysine, do not behave in a similar manner. The bundling activity is insensitive to ATP. By assaying bundling activity with dark–field microscopy and employing std. biochem. procedures, a small no. of polypeptides involved in the bundling process were identified. Affinity–isolated antibodies to one of these polypeptides (M_r = 76,000) were found to co–localize with MTs in the cortical array of protoplasts. The importance of these proteins in the cell and to their relationship to microtubule–assocd. proteins in other eukaryotes are discussed.

110: 151371n Phosphoribosyl pyrophosphate and the measurement of inorganic pyrophosphate in plant tissues. Dancer, Jane E.; Ap Rees, Tom (Bot. Sch., Univ. Cambridge, Cambridge, UK CB2 3EA). *Planta* 1989, 177(2), 261–4 (Eng). Plants contain appreciable amts. of inorg. pyrophosphate (PPi), and the breakdown of phosphoribosyl pyrophosphate (PPRibP) does not contribute significantly to the PPi detected in plant exts. Inorg. pyrophosphate in exts. of the roots of *Pisum sativum*, clubs of the spadices of *Arum maculatum*, and the developing endosperm of *Zea mays* was assayed with pyrophosphate fructose 6–phosphate 1–phosphotransferase (EC 2.7.1.90), and with sulfate adenyltransferase (EC 2.7.7.4). The two different assays gave the same value for PPi content, and for recovery of added PPi. PPRibP was converted to PPi during the extn. of PPi. However, the amts. of PPRibP in clubs of *A. maculatum* and the developing endosperm of *Z. mays* were negligible in comparison with the contents of PPi.

110: 151372p Incorporation of cysteine and selenocysteine into cystathionine and selenocystathionine by crude extracts of spinach. Dawson, J. C.; Anderson, J. W. (Bot. Dep., La Trobe Univ., Bundoora, 3083 Australia). *Phytochemistry* 1988, 27(11), 3453–60 (Eng). Crude exts. of spinach catalyzed the incorporation of [^{3}H]cysteine into cystathionine in the presence of *O*–phosphoryl–homoserine (PHS) or *O*–succinylhomoserine (SHS). They also supported the incorporation of [U–^{14}C]PHS into cystathionine in the presence of cysteine. These reactions were completely inhibited by 0.1 mM propargylglycine and 1 mM amino–oxyacetate and less strongly by vinylglycine, β–cyanoalanine, and isonicotinic acid hydrazide. These properties are consistent with the synthesis of cystathionine by cystathionine γ–synthase (CγS) (EC 4.2.99.9) activity. Spinach exts. also incorporated [^{75}Se]selenocysteine into selenocystathionine in the presence of SHS in a reaction which was sensitive to propargylglycine. In the presence of selenocysteine, spinach exts. incorporated [^{14}C]PHS into a compd. with chromatog. characteristics indistinguishable from selenocystathionine at a rate similar to that for cysteine. When cysteine and selenocysteine were supplied together the amt. of [^{14}C]PHS incorporated was slightly less than for either substrate alone. Conversely, selenocysteine strongly inhibited the incorporation of [^{3}H]cysteine into cystathionine. Thus, selenocysteine acts as an alternative substrate of CγS cysteine resulting in the formation of the selenium isolog of cystathionine. In crude spinach exts., CγS exhibits a greater affinity for selenocysteine (K_m ~70 μM) than cysteine (K_m ~240 μM).

110: 151373q Carbon balance during CAM: an assessment of respiratory carbon dioxide recycling in the epiphytic bromeliads Aechmea nudicaulis and Aechmea fendleri. Griffiths, H. (Dep. Biol., Univ. Newcastle upon Tyne, Newcastle upon Tyne, UK NE1 7RU). *Plant, Cell Environ.* 1988, 11(7), 603–11 (Eng). The regulation of crassulacean acid metab. (CAM) under controlled environmental conditions was investigated for 2 tropical epiphytes. *A. fendleri* is restricted to wet, upper montane regions of Trinidad, while *A. nudicaulis* has a wider distribution extending into more arid regions. The 2 species differ in expression of CAM, since the proportion of respiratory CO_2 recycled as part of CAM was found to be much lower in *A. fendleri*. This study compared the efficiency of water use and role of respiratory CO_2 recycling under 2 photosynthetically active radiation (PAR) regimes (300 and 120 μmol m^{-2} s^{-1}) and 3 night temps. (12, 18 and 25°). Dark CO_2 uptake rates for both species were comparable to plants in the field (max. of 2.3 μmol m^{-2} s^{-1}). Total net CO_2 uptake at night increased on leaf area basis with temp. for both species under higher PAR, although under the low PAR regime CO_2 uptake was maximal at 18°. Water–use efficiency increased at 18° and 25° during dark CO_2 uptake and also during late afternoon photosynthesis in both species. For *A. fendleri*, dawn to dusk changes in titrable acidity (ΔH^+) were similar under high and low PAR, although ΔH^+ was correlated to night temp. and PAR in *A.*

enone system. The molecule is not planar, the angle between the planes of the phenyl rings being 13.1 (4)°. Intermolecular O—H—O bonds with distances O(6A)—O(2A) = 2.689 (2), H(2A)—O(6A) = 1.86 (4) Å and angle O(2A)—H—O(6A) = 176 (4)° form endless chains of molecules along b.

139174. URLICH, GERD, KARL HEINZ KLASKA*, OTTO H. JARCHOW*, HELMUT W. SCHMALLE, WILFRIED A. KOENIG, HOLGER HEITSCH, RALPH RATHMANN, BJORN M. HAUSEN and KARL-HEINZ SCHULZ. (Anorganisch-Chemisches Inst., Universitaet Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.) ACTA CRYSTALLOGR SECT C CRYST STRUCT COMMUN 46(9): 1715–1718. 1990. **Structure of 2-methoxy-6-nonyl-1,4-benzoquinone, a synthetic contact allergen related to the naturally occurring primin.**—$C_{16}H_{24}O_3$ M_r = 264.36, triclinic, $P\bar{1}$, a = 4.167, b = 9.658(1), c = 19.093(1) Å, α = 89.66(1), β = 87.27(1), γ = 79.07(1)°, V = 753.65(1) Å^3, Z = 2, D_x = 1.165 Mg m^{-3}, λ(Cu Kα) = 1.5418 Å, μ = 0.595 mm^{-1}, F(000) = 288, T = 295 K, R is 0.047 for 1682 observed unique reflections. The angle between the quinone ring plane and the mean plane defined by the aliphatic nonyl chain atoms is 17.4(3)°. The average C_{sp^2} — C_{sp^3} bond distance and corresponding angle of the side chain are 1.522 (3) Å and 113.2(2)°. The average dimensions of the quinone ring are C — C 1.485(3), C = C 1.337(3), C = O 1.209(3) Å, C — C — C 118.4(2), C = C 120.8(2)°. Neighboring molecules form dimers across centres of symmetry which are linked by C — H · · · O hydrogen bonds with H(3) · · · O(4') 2.42(4), C(3) · · ·O(4') 3.299(2) Å, and angle C(3)–H(3) · · · O(4') 164(3)°. The dimers are held together by van der Waals forces between the nonyl side chains, and by C(16) — H · · · O(2") hydrogen bonds, with H(163) · · · O(2") 2.56(4), C(16) · · · O(2") 3.357 (3) Å, and angle C(16) —H(163) · · · O(2") 140(3)° [(i) ¯x, 1¯y, ¯z; (ii) 1¯x, ¯y, ¯z].

139175. ARNDT, ROLF, JAN-ERIC BERG and INGER WAHLBERG*. (Reserca AB, P.O. Box 17007, S-104 62 Stockholm, Sweden.) ACTA CHEM SCAND 44(8): 814–825. 1990. **Tobacco chemistry: 71. Structure determination and biomimetic studies of five new tobacco cembranoids.**—Five new diterpenoids of the cembrane class have been isolated from flowers of Greek tobacco. They have been identified as (1S,2E,4S,6R,7R,11S)–2,8(19),12(20)–cembratriene–4,6,7,11–tetraol (1) and the corresponding (7S)–epimer (2), (1S,2E,4S,8S,11S)–4,8,11–trihydroxy–2,12(20)–cembradien–6–one (3) and the corresponding (8R)–epimer (4) and (1S,2E,4S,6R,7E,11S,12R)–2,7–cembradiene–4,6,11,12–tetranol (5) by spectral methods and biomimetic syntheses (1–4). The outcome of the reaction of the (4S,6R,11S)–triol 10 and the 6–oxo–(4S,8S)–diol 14 with singlet oxygen is discussed as is the biogenesis of the new compounds (1–5). The crystal structures of the tetraol 1 and oxotriol 16 have been determined and are described.

139176. USMANI, J. N.*, MAHBOOB A. KALHORO and SHAHNAZ ISMAIL. (Scientific Information Centre PCSIR, Saddar, Karachi.) PAK J SCI IND RES 33(1/2): 37–38. 1990. **The determination of thiamine content in seaweeds.**—Determination of Thiamine in Seaweeds was carried out using improved technique with the help of three different Spectrophotometers. This method was applied on standard sample of thiamine (98.5–101.5%) and seven different species of Seaweeds belonging to Red and Brown family: *Botryocladia microphysa, Carpogonia florideae, Dictyota dichotoma, Tetrasporangia, Iyengaria stellata, Samia indica* and *Hypnea musciformis*. The method was also compared with other methods such as U.S.P., B.P., Pak. Pharmacopeae.

139177. KALHORO, MAHBOOB A. and J. N. USMANI. (PCSIR Laboratories Complex, Karachi–39, Pakistan.) PAK J SCI IND RES 33(1/2): 64–65. 1990. **Study of vitamins in selected seaweeds of Karachi coast [Pakistan].**—Eight different species of seaweeds belonging to Rhodophytes and Phaeophytes family –*Tetra sporangia* (Padina), *Botryocladia microphysa, Carpogonia floridease, Dictyota dichotoma, Iyengaria stellata, Samia indica, Hypnea musciformis* and *Sargassum vulgare* were studied for their contents of vitamins.

139178. HU, B. H.*, Y. L. LIU*, T. ZHANG* and W. Z. SONG. (Institute Medicinal Plant Development, Chinese Academy Medical Sciences, Beijing 100094.) ACTA PHARM SIN 25(4): 302–306. 1990. [In Chin. with Engl. summ.] **Studies on the structure of scuteamoenin from the root of *Scutellaria amoena*.**—In continuing our studies on the flavonoids from *Scutellaria amoena* C. H. Wright, a new flavanone (I) and six known compounds (II ~ VII) were isolated from the roots of this plant. On the basis of spectroscopic analysis (UV, ^{1}H NMR, ^{13}C NMR, MS and CD) and chemical evidence, the structure of the new compound was elucidated as (2S)–2',5,6'–trihydroxy–7–methoxyflavanone (I) and named scuteamoenin, the other six known compounds were identified as (2R, 3R) –3,5,7–trihydroxyflavanone (II), 2',3,5,6',7–pentahydroxyflavone (III), 2',5,7–trihydroxy–6–methoxyflavone (IV), skullcaflavone II (V), chrysin (VI) and β–sitosterol (VII) respectively. Compounds II–VII were obtained from this plant for the first time.

139179. WILIMOWSKA-PELC, ANNA. (Inst. Biochem., Univ. Wroclaw, Tamka 2, 50–137 Wroclaw, Pol.) ACTA SOC BOT POL 58(4): 563–574. 1989(1990). [In Engl. with Engl. and Pol. summ.] **Trypsin inhibitors in turnip (*Brassica rapa* L.) seeds.**—A method of trypsin inhibitors isolation from turnip seeds is described. Inhibitors were extracted with 0.01 N HCl, concentrated by salting out with ammonium sulfate, and purified using ion–exchange chromatography on Sp–Sephadex C–25, QAE–Sephadex A–25 and affinity chromatography on immobilized trypsin. Among the three isolated inhibitors ITR I of molecular weight 15.9 kDa, pI. 6.4, inhibited trypsin activity only. Inhibitors ITR II and ITR III inhibited also chymotrypsin activity, they had similar molecular weight (about 10 kDa), but their pI is 7.5 and over 10, respectively. Arginine residue occurred in P_1 position of the reactive site of inhibitors ITR I and ITR III, while in ITR II this position was occupied by lysine residue. Electrophoresis on polyacrylamide gel revealed that each inhibitor possessed two protein fractions, probably a virgin and modified form, with the reactive site peptide bond broken by trypsin.

139180. WEINGES, KLAUS* and ULRICH LERNHARDT. (Organische–Chem. Inst. der Univ. Heidelberg, Im Neuenheimer Feld 270, D–6900 Heidelberg.) LIEBIGS ANN CHEM 0(8): 751–754. 1990. [In Ger. with Engl. summ.] **Chemistry and stereochemistry of iridoids: XIII. Synthesis of enantiomerically pure methyl (1R,2S,2'Z)–(dextro)–jasmonate starting from catalpol.**—Enantiomerically pure methyl (1R,2S,2'Z)–(+)–jasmonate (2), which was recognized as a component of the fragrance of the jasmine flower oil, was synthesized starting from catalpol (3). 2 is easily epimerized to 1. Thus, it is necessary to use very mild conditions in the course of the synthesis and purification. These results raise the question whether 2 exists as a natural product in the flower of jasmine 1 arises during the isolation.

139181. GUNAWAN, SONNY, BERT STEFFAN and WOLFGANG STEGLICH* (Inst. Organische Chemie and Biochem. der Univ. Bonn, Gerhard–Domagk–Strasse 1, D–5300 Bonn 1.) LIEBIGS ANN CHEM 0(8): 825–828. 1990. [In Ger. with Engl. summ.] **Xylaral, a hydroxyphthalide derivative from fruiting bodies of *Xylaria polymorpha* (Ascomycetes).**—Fruiting bodies of Dean Man's Fingers, *Xylaria polymorpha*, contain a hydroxyphthalide derivative, xylaral (1), which exhibits a violet color reaction with aqueous ammonia. 1 is a homolgue of cyclopaldic acid (5) with a C_{14}–side chain containing an enone moiety.

139182. PEI, Y. H., X. LI, T. R. ZHU and L. J. WU. (Research Dep. Natural Drugs, Shenyang College Pharmacy, Shenyang 110015.) ACTA PHARM SIN 25(4): 267–270. 1990. [In Chin. with Chin. and Engl. summ.] **Studies on the structure of a new flavanonol glucoside of the root sprouts of *Agrimonia pilosa* Ledeb.**—Three compounds were isolated from benzene and acetone extracts of the root–sprouts of *Agrimonia pilosa* Ledeb. On the basis of physicochemical properties, spectroscopy (UV, IR, NMR, MS, CD, GC) and chemical degradation, two known compounds were identified as palmitic acid (VIII) and daucosterol (IX), one new compound was elucidated as (2S,3S)–(–)–taxifolin–3–O–β–D–glucopyranoside (X).

139183. ZHAO, Y. Q.*, C. L. YUAN*, Y. Q. FU*, X. J. WEI*, H. J. ZHU*, Y. J. CHEN, L. J. WU and X. LI. (Liaoning College Traditional Chinese Medicine, Shenyang 110032.) ACTA PHARM SIN 25(4): 299–301. 1990. [In Chin. with Engl. summ.] **Chemical studies of minor triterpene compounds isolated from the stems and leaves of *Panax ginseng* C.A. Meyer.**—Two new natural products were isolated from the stems and leaves of *Panax ginseng* C.A. Meyer cultivated in Liaoning, China. Their structures were characterized as 20(R)–dammaran–3β,6α,12β,20,25–pentol and 20(R)–dammaran–3β,6α,12β, 20,25–pentol–6–O–α–L–rhampyranosyl(1 → 2)–O–β–D–glucopyranoside.

139184. JURZYSTA, MARIAN*, KEITH PRICE, CARALYN RIDOUT and ROGER FENWICK. (Inst. Soil Sci. and Plant Cultivation, Dep. Biochem. and Crop Quality, 24–100 Pulawy, Pol.) ACTA SOC BOT POL 58(4): 575–582. 1989(1990). [In Engl. with Engl. and Pol. summ.] **The structures of four triterpenoid saponins isolated from the seed of *Trifolium incarnatum*.**—A novel bisdesmosidic saponin, 3–O–[α–L–rhamnopyranosyl(1 → 2)–β–D–galactopyranosyl(1 → 2)–β–D–glucuronopyranosyl]–22–O–[β–D–glucopyranosyl(1 → 2)–β–D–glucopyranosyl] olean–12–ene–24–ol has been isolated from the seed of the clover, *Trifolium incarnatum*. The structure of this and of three other structurally related saponins, 3–O–(β–D–glucuronopyranosyl)olean–12–ene–22, 24–diol, soyasaponin I and soyasaponin III have been assigned on the basis of spectral and chemical evidence.

139185. FUKUSHIMA, AKIYOSHI, HIROAKI HASE and KOSHI SAITO*. (Dep. Applied Chem., Fac. Eng., Tokai Univ., 1117 Kitakaname, Hiratsuka–shi, Kanagawa–ken 259–12, Jpn.) ACTA SOC BOT POL 58(4): 583–592. 1989(1990). [In Engl. with Engl. and Pol. summ.] **Further studies on the adsorption of plant phenols by synthetic polymers.**—Pyrocatechol, catechol, caffeic acid, chlorogenic acid, safflor yellow A, safflor yellow B, precarthamin and carthamin were effectively adsorbed by insoluble polyvinyl–N–pyrrolidone (PVP) in a neutral buffer solution. These eight phenols also bound with Amberlite XAD resins, however, the rate was found to be far less efficient than that of PVP. The average rate of the phenol binding was calculated as following order (%): PVP (42.7), Amberlite XAD–2 (16.6), Amberlite XAD–4 (10.1), Amberlite XAD–7 (13.0), Amberlite XAD–8 (17.7). No 3,4–dihydroxyphenylalanine was adsorbed by PVP, while the O–dihydroxylic acid could be removed by Amberlite XAD–4, XAD–7 and XAD–8. Data from using different weights of the test polymers showed that the rate of the phenol adsorption rose in proportion to each increasing amount of the adsorbents. PVP also admittedly maintained its predominant capacity for phenol binding over that of each member of the Amberlite resins.

139186. MOTL, O.*, G. OCHIR* and K.–H. KUBECZKA. (Inst. Organic Chem. Biochemistry, Czechoslovak Academy Sci., 166 10 Prague 6, Czechoslovakia.) FLAVOUR FRAGRANCE J 5(3): 153–156. 1990. **Composition of *Achillea asiatica* Serg. essential oil.**—The essential oil obtained from *Achillea asiatica* Serg. was analysed by means of capillary gas chromatography and various physico–chemical methods; 40 components have been identified. The composition of *Achillea millefolium* L. essential oil is very similar.

139187. MIYAZAWA, MITSUO and HIROMU KAMEOKA. (Dep. Applied Chemistry, Fac. Sci. Engineering, Kinki Univ., Kowakae, Higashiosaka–shi, Osaka 577, Japan.) FLAVOUR FRAGRANCE J 5(3): 157–160. 1990. **Volatile flavor components of glycyrrhizae radix (*Glycyrrhiza glabra* var. *glandulifera* Regel et Herder) from China.**—The composition of the volatile oil of Kanzou from *Glycyrrhiza glabra* L. var. *glandulifera* Regel et Herder growing in northeast China has been investigated by capillary GC, GC–MS and ^{1}H–NMR. The oil contained 81 volatile components of which 35.0% were terpenoid. The main constituents were octanoic acid (11.4%), paeonol (8.9%), octadecane (8.6%), benzaldehyde (7.5%), α–terpineol (7.5%) and 4–terpineol (7.2%).

Aspergillus niger has been purified 1400-fold by ammonium sulphate fractionation, ion exchange chromatography, hydrophobic interaction chromatography and gel filtration. The enzyme consisted of two identical subunits, each with a molecular weight of 60,000. The isoelectric point was 4.5. The optimal pH for the hydrolysis of cinnamic acid ethyl ester and 2-furylacryloyl N-hydroxy succinimide ester was between 5 and 7. The enzyme, which had no lipase activity, catalyzed the hydrolysis of activated ester substrates where the alcohol moiety was N-hydroxysuccinimide, p-nitrophenol and phenol as well as, with lower rates, unactivated esters like ethyl and benzyl esters. The enzyme exhibited a preference for substrates with an acyl moiety containing an aryl group. The enzyme was inhibited by PMSF but not by Hg^{++} and EDTA. It is classified as a serine carboxylesterase.

134724. SVENDSEN, I. B.*, DIANA NICOLOVA, IVAN GOSHEV and NICOLAY GENOV. (Dep. Chem., Carlsberg Lab., Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby.) CARLSBERG RES COMMUN 54(6): 231-240. 1989. **Isolation and characterization of a trypsin inhibitor from the seeds of kohlrabi (*Brassica napus* var. *rapifera*).**—A trypsin inhibitor with a K_m of 5×10^{-9} M has been isolated from kohlrabi (*Brassica napus* var. *rapifera*). Subtilisin DY is inhibited only weakly and chymotrypsin not at all. The inhibitor is closely related to napin as determined by amino acid sequence analysis which also showed the inhibitor to be polymorphous. The inhibitor has been further characterized by means of molecular weight determination using SDS gel-electrophoresis and by amino acid analysis, fluorimetry as well as circular dichroism. A simplified method for purification of napins is given.

134725. HRMOVA, MARIA, EVA PETRAKOVA and PETER BIELY. (Inst. Chem., Slovak Academy Sci., Dubravska cesta 9, 842 38 Bratislava, Czechoslovakia.) J GEN MICROBIOL 137(3): 541-548. 1991. **Induction of cellulose-degrading and xylan-degrading enzyme systems in *Aspergillus terreus* by homodisaccharides and heterodisaccharides composed of glucose and xylose.**—Synthetic heterodisaccharides composed of glucose and xylose were tested as inducers of cellulose- and xylan-degrading enzymes in *Aspergillus terreus*, and the inducing abilities were compared with those of sophorose and xylobiose or their positional isomers. Measurement of secreted and cell-associated enzyme activities revealed that the heterodisaccharides induced the synthesis of the cellulolytic and xylanolytic enzymes. 2-O-β-D-glucopyranosyl D-xylose (Glcβ1-2Xyl) being the most powerful inducer. Sophorose and 2-O-β-D-xylopyranosyl D-xylose (Xylβ1-2Xyl), or their positional isomers, selectively induced the synthesis of cellulases and β-xylanases, respectively. An analysis of the extracellular enzymes (which were separated by isoelectric focusing followed by detection using chromogenic and fluorogenic substrates) showed that Glcβ1-2Xyl initiated the synthesis of specific endo-1,4-β-glucanases and specific endo-1,4-β-xylanases identical to those produced separately in response to sophorose or Xylβ1-2Xyl. Glcβ1-2Xyl also induced specific endo-1,4-β-glucanases that hydrolysed 4-methylumbelliferyl β-lactoside at the agluconic bond. The results strengthen the concept of separate regulatory control of the synthesis of cellulases and β-xylanases. The results also suggest that mixed disaccharides, composed of glucose and xylose moieties, which may occur in nature, could play an important role in regulating the synthesis of wood-degrading enzymes.

134726. VIEGAS, CRISTINA A. and ISABEL SA-CORREIA. (Lab. Engenharia Bioquimica, Inst. Superior Tecnico, Av. Rovisco Pais, 1096 Lisboa, Codex, Portugal.) J GEN MICROBIOL 137(3): 645-652. 1991. **Activation of plasma membrane ATPase of *Saccharomyces cerevisiae* by octanoic acid.**—Plasma membrane ATPase activity of *Saccharomyces cerevisiae* IGC 3507/III grown in the presence of the lipophilic acid octanoic acid [4-50 mg l^{-1} (0.03-0.35 mM), pH 4.0] was 1.5-fold higher than that in the cells grown in its absence. The K_m for ATP, the pH profile and the sensitivity to orthovanadate of the basal and the activated forms of the membrane ATPase were identical. This activation was closely associated with a decrease in the biomass yield and an increase in the ethanol yield, and was rapidly reversed in vivo after removal of the acid. However, the activated level was preserved when membranes were extracted and subjected to manipulations which eliminated or decreased octanoic acid incorporation in the plasma membrane. The activity of the basal plasma membrane ATPase in the total membrane fraction was slightly increased by incubation at pH 6.5 with octanoic acid at 100 mg l^{-1} or less (2.4 mg acid form plus 97.6 mg octanoate ion l^{-1}). However, destruction of the permeability barrier between the enzyme and its substrate could not explain the in vivo activation. A role for plasma membrane ATPase activation in the regulation of the intracellular pH (pH_i) of cells grown with octanoic acid was not proven.

134727. RAKSHIT, S. K.* and V. SAHAI. (Dep. Chem. Eng., Indian Inst. Technol., Madras-600 036, India.) BIOPROCESS ENG 6(3): 101-108. 1991. **Optimal control strategy for the enhanced production of cellulase enzyme using the new mutant *Trichoderma reesei* E-12.**—Cellulase enzyme production was enhanced using the mutant strain Trichoderma reesei E-12, which was shown to be partially resistant to catabolite repression. An optimal profile for pH, which was found to be the critical environmental parameter, was determined using a rigorous mathematical optimization procedure. Semi-empirical models were used to minimize complications in the computation. A 30% increase in enzyme activity and productivity was obtained using the optimal pH strategy as compared to the pH cycling strategy.

134728. HAMMER, THOMAS, RUDIGER BODE and DIETER BIRNBAUM. (Inst. Biochemie, Fachrichtung Biol., Ernst-Moritz-Arndt-Univ. Greifswald, 2200 Greifswald, Ger.) J GEN MICROBIOL 137(3): 711-716. 1991. **Occurrence of a novel yeast enzyme, L-lysine ε-dehydrogenase, which catalyzes the first step of lysine catabolism in *Candida albicans*.**—The yeast *Candida albicans* is able to utilize L-lysine as the sole nitrogen and carbon source accompanied by intracellular accumulation of α-aminoadipate-δ-semialdehyde. A novel yeast amino acid dehydrogenase catalyzing the oxidative deamination of the ε-group of L-lysine was found in this yeast. The enzyme, L-lysine ε-dehydrogenase, is strongly induced in cells grown on L-lysine as the sole nitrogen source. The enzyme is specific for both L-lysine and $NADP^+$. The K_m values were determined to be 0.87 mM for L-lysine and 0.071 mM for $NADP^+$. An apparent M_r of 87,000 was estimated by gel filtration. The enzyme has maximum activity at pH 9.5 and a temperature optimum of 32° C under our assay conditions.

134729. TABLERO, MARISELA, ROBERTO ARREGUIN, BARBARIN ARREGUIN, MANUEL SORIANO, ROSA I. SANCHEZ, ADELA RODRIGUEZ ROMERO* and ANDRES HERNANDEZ-ARANA. (Inst. Quimica, Univ. Nac. Autonoma Mex., Circuito Exterior, Ciudad Universitaria, Coyoacan 04510 Mexico D.F., Mex.) PLANT SCI (LIMERICK) 74(1): 7-16. 1991. **Purification and characterization of multiple forms of asclepain *g* from *Asclepias glaucescens* HBK.**—Ten asclepain forms were found to be present in the latex of milkweed (*Asclepias glaucescens* H.B.K.) plants. Four of them were purified by high performance liquid chromatography on a cation exchange column, and characterized. Asclepains Ag_1, Ag_2, Ag_3 and Ag_4 were isolated as homogeneous proteins of very similar molecular weights (approx. 23000) with isoelectric points greater than 9.0. These forms possess nearly identical secondary structures, as judged from their circular dichroism spectra. Interestingly, one of these forms (Ag_1) markedly differed from the other three forms with respect to its specificity toward ester and amide substrates.

134730. ESEN, ASIM* and CUMHUR COKUMS. (Dep. Biol., Va. Polytechnic Inst. State Univ., Blacksburg, Va. 24061, USA.) PLANT SCI (LIMERICK) 74(1): 17-26. 1991. **pH-dependent and temperature-dependent β-glucosidase multiplicity in maize (*Zea mays* L.) is a proteolysis artifact.**—Maize β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) isolated from inbred lines displays multiple electrophoretic variants in a time-dependent manner during storage, especially at low pH (below pH 6.0) and in the presence of a reducing agent. The enzyme was extracted from the coleoptiles of 5-6-day-old maize seedlings with a variety of aqueous buffers and assayed for activity spectrophotometrically and by zymogram techniques. The pH values of the crude enzyme preparations were adjusted to vary from 3.0 to 10.0 and the preparations were incubated at different temperatures (-30°, 4°, 25° and 37° C) for varying lengths of time in the presence of the reducing agent 2-mercaptoethanol (2-ME) and assayed for activity and changes in zymogram patterns. The results showed that the enzyme lost activity at higher temperatures (25° and 37° C) and at pH values < 4 and > 9. Similarly, the number of charge variants resolved by electrophoresis increased during incubation, especially at higher temperatures. These novel variants had increased anodal mobilities; they could be resolved into 5 distinct bands after extracts were incubated at pH 4-6. When polypeptide profiles were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the staining intensity of individual polypepides decreased and complete loss of some polypeptides occurred between pH 4 and 6 in a time-dependent manner, especially after incubation at 25° and 37° C. Based on these data, it was concluded that maize β-glu multiplicity observed in zymograms of inbred lines is an artifact of proteolysis. An SH-proteinase active under acidic conditions and requiring a reducing agent (e.g., 2-ME) for activation was postulated to be primarily responsible for artifactual β-glu multiplicity. Another class (undefined) of proteolytic activity was responsible for producing charge multiplicity above pH 6; but this multiplicity occurred as a broad and diffuse zone instead of distinct bands.

134731. OJHA, MUKTI* and BERTRAND FAVRE. (Lab. General Microbiol., Dep. Botany Plant Biol., Univ. Geneva, Sci. III 30, Quai Ernest-Ansermet 1211, Geneve 4, Switz.) PLANT SCI (LIMERICK) 74(1): 35-44. 1991. **In vitro and in vivo phosphorylation of calpain-like protease of *Allomyces arbuscula*.**—The calpain-like protease from *Allomyces arbuscula* was phosphorylated in vitro by a serine/threonine protein kinase from *Neurospora crassa* typical of casein kinase II. The protease contained covalently bound phosphate when the culture was labelled in vivo with $^{32}P_i$. The phosphoryl linkage was sensitive to alkaline phosphatase. Phosphoserine was the major phosphohydroxylamino acid detected after acid hydrolysis of the in vitro and in vivo phosphorylated enzyme. Phosphopeptide maps of the protease phosphorylated either in vitro or in vivo were similar. These data suggest that in vivo the protease may be regulated by phosphorylation-dephosphorylation mechanisms.

134732. REN, LIFEN, JOHANN SALNIKOW* and JOACHIM VATER. (Technische Univ. Berlin, Inst. Biochemie Molekulare Biologie, Franklinstr. 29, D-1000 Berlin 10, Ger.) PLANT SCI (LIMERICK) 74(1): 1-6. 1991. **Multiple forms of the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase in maize and spinach.**—Native small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) from maize has been electrophoretically resolved into five isoforms of identical molecular weight possessing, however, different isoelectric points (pI = 7.4, 7.2, 6.5, 5.7 and 5.2). Gene expression studies (Sheen and Bogorad, EMBO J., 5 (1986) 3417) and proteinchemical sequencing (Ren et al., Biol. Chem. Hoppe-Seyler, 369 (1989) 609) suggest that the observed isoforms have slightly different amino acid sequences as a result of a multigen expression. For spinach rubisco the native small subunit resolves into two isoforms with pI = 6.6 and 7.2.

134733. GRAFI, GIDEON*, ERNA MELLER, NAAMIT SHER and ILAN SELA. (Virus Lab., Otto Warburg Center Biotechnology, Hebrew Univ., Fac. Agric., Rehovot 76100, Isr.) PLANT SCI (LIMERICK) 74(1): 107-114. 1991. **Characterization of S1/-mung-bean-type nuclease activity in plant cell suspensions.**—A nuclease activity was detected in cultured cells of various plants. The enzyme is excreted into the growth medium and degrades single-treated forms of nucleic acids. This activity has been characterized as an S1/mung-bean-type nuclease, also by its ability to cleave supercoiled DNA only at specific sites. The growth media conditions (low pH and metal ions) favor the activity of this nuclease, while ethylenediamine tetraacetic acid (EDTA) inhibits its activity and polyethylene glycol (PEG) slows the reaction. This activity should be taken into account when transforming plant cells since RNA degrades almost immediately and plasmids are linearized when incubated with the cells. This is especially important since the effect of the topology of certain DNA regions on gene expression is well